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Antiarrhythmogenic Effect of Reconstituted High-Density Lipoprotein Against Ischemia/Reperfusion in Rats

Satoshi Imaizumi, MD,* Shin-ichiro Miura, MD, PHD,* Kazuto Nakamura, MD, PHD,†‡ Yoshihiro Kiya, MD,* Yoshinari Uehara, MD, PHD,* Bo Zhang, PHD,* Yoshino Matsuo, PHD,* Hidenori Urata, MD, PHD,§ Munehito Ideishi, MD, PHD,* Kerry-Anne Rye, PHD,‖ Masataka Sata, MD, PHD,†‡ Keijiro Saku, MD, PHD, FACC*

Fukuoka, Tokyo, and Chikushino, Japan; and Sydney, Australia

Objectives This study analyzed the antiarrhythmogenic effect of reconstituted high-density lipoprotein (rHDL) against ischemia/reperfusion in vivo.

Background Recent studies have suggested that a reduction in the plasma HDL level may contribute to cardiac sudden death. Although there are currently only a few therapeutic strategies for increasing HDL, an exciting new therapeutic option, rHDL, has recently been developed to prevent coronary artery disease.

Methods To analyze the suppression of reperfusion arrhythmia by rHDL (apolipoproteinA-I with 1-palmitoyl-2-oleoyl-phosphatidyl-choline), 92 male Wistar rats were divided into 10 groups: rats that had been pre-treated with or without rHDL, apolipoproteinA-I, or 1-palmitoyl-2-oleoyl-phosphatidyl-choline in the presence or absence of inhibitors of Akt protein kinase, nitric oxide (NO), or extracellular-signal-regulated kinase (ERK) administered intravenously before left coronary artery occlusion. We also used human coronary artery endothelial cells and adenosine triphosphate-binding cassette transporter (ABC) A1-, ABCG1-, or scavenger receptor class B, type I–transfected ldlA7 cells systems.

Results The duration of ventricular tachycardia or ventricular fibrillation after reperfusion in rHDL–pre-treated rats was much shorter than that in untreated rats. ApolipoproteinA-I or 1-palmitoyl-2-oleoyl-phosphatidyl-choline alone had no effect. The effect of rHDL was blocked by inhibitors of Akt, NO, and ERK. Plasma NO concentration in the rHDL group was significantly higher. In addition, rHDL activated phospho(p)-Akt, p-ERK, and p-endothelial NO synthesis in endothelial cells. The rHDL activated p-ERK in ABCA1- or ABCG1-transfected but not scavenger receptor class B, type I–transfected ldlA7 cells.

Conclusions The rHDL-induced NO production, probably mediated by ABCA1 or ABCG1 through an Akt/ERK/NO pathway in endothelial cells, may suppress reperfusion-induced arrhythmias. The HDL-based therapy may hold the promise of reducing the incidence of such arrhythmias after ischemia/reperfusion. (J Am Coll Cardiol 2008;51:1604–12) © 2008 by the American College of Cardiology Foundation

Plasma high-density lipoprotein (HDL) protects against cardiac events by mediating cholesterol efflux from the arterial wall (reverse cholesterol transport), thereby preventing the formation of atherosclerotic plaque in coronary arteries (1). Recent studies have suggested that a reduction in the plasma HDL level may contribute to cardiac sudden death (2). This contribution of HDL must be related to reverse cholesterol transport and the stabilization of vulnerable, unstable plaque (3). There are several reports that suggest a direct relation between plasma high- and low-density lipoproteins and fatal arrhythmia (4,5). Furthermore, HDL has many pleiotropic effects (1), such as antioxidant, anti-inflammatory, and antithrombotic properties, in addition to its ability to enhance reverse cholesterol transport. Although HDL is a target in the treatment of atherosclerotic coronary artery disease, there are currently only a few therapeutic strategies for increasing HDL, such as statins and cholesterol ester transfer protein inhibitors. However, an exciting new therapeutic option, reconstituted HDL (rHDL), has recently been developed and is currently

From the *Department of Cardiology, Fukuoka University School of Medicine, Fukuoka, Japan; †Department of Cardiovascular Medicine, and the ‡Department of Advanced Clinical Science and Therapeutics, University of Tokyo Graduate School of Medicine, Tokyo, Japan; §Department of Cardiovascular Diseases, Fukuoka University Chikushi Hospital, Chikushino, Japan; and the ‖Lipid Research Group, Heart Research Institute, Camperdown, Sydney, New South Wales, Australia. Supported in part by a grant (18590826) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Central Research Institute of Fukuoka University, Japan.

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the focus of intriguing research (6). Although pleiotropic effects of rHDL may protect isolated rat hearts against an ischemia/reperfusion (I/R) injury that involves the reduction of tumor necrotic factor-α and the enhancement of prostaglandin release (7), the antiarrhythmogenic effect of rHDL against I/R remains unclear in vivo.

Nitric oxide (NO) is an endogenous regulatory molecule that is involved in a variety of physiological activities such as the regulation of blood pressure (BP). A previous in vitro study examined HDL-stimulated NO release (1). In addition, NO is also known to trigger ischemic preconditioning against I/R arrhythmia (8). For instance, an angiotensin receptor blocker induced an antiarrhythmogenic effect (9) that may be related to NO production in animal I/R models (10). Because NO is a key mediator for antiarrhythmogenic effects, HDL-induced NO production may protect the heart from cardiac injury during I/R via effects on both cardiac tissue and coronary perfusion. Therefore, we hypothesized that rHDL contributed to the prevention of I/R arrhythmia through its pleiotropic effects, such as NO production in endothelial cells (ECs). In this study, we analyzed the antiarrhythmogenic effect of rHDL using a rat I/R arrhythmia model and cell systems.

Methods

Materials. The following antibodies and reagents were purchased or kindly provided: PD98059, a specific inhibitor of extracellular-signal-regulated kinase (ERK) (Cell Signaling Technology Inc, Danvers, Massachusetts); N-nitro-L-arginine methyl ester hydrochloride (L-NAME), a specific inhibitor of endothelial NO synthase (eNOS) (Sigma-Aldrich Co., St. Louis, Missouri); wortmannin, a specific inhibitor of phosphoinositide 3 (PI3) kinase (Sigma-Aldrich); antibodies for Akt, phospho(p)-Akt, ERK1/2, and p-ERK1/2 (Thr^{202}/Tyr^{204}) (Cell Signaling Technology).

Preparation of rHDL. Discoidal rHDL containing 1-palmitoyl-2-oleoyl-phosphatidyl- choline (POPC) (Avanti Polar Lipids, Alabaster, Alabama) and apolipoproteinA-I (A-I) (initial POPC/A-I molar ratio 100/1) was prepared by the cholate dialysis method as described previously (11).

Animal preparation. Male Wistar rats (250 to 350 g) were anesthetized with 1.5% pentobarbital (60 mg/kg) intraperitoneally. Subdermal electrodes were placed to allow the determination of a lead II electrocardiogram (ECG). Myocardial ischemia was induced by temporary occlusion of the left main coronary artery. After tracheotomy, the animals were ventilated with room air by a respirator for small rodents (model 683; Harvard rodent ventilator, Harvard Apparatus, Inc., Holliston, Massachusetts). The chest was opened by a left thoracotomy and the heart was exposed. After incision of the pericardium to allow access to the left main coronary artery, the hearts were subjected to 5 min of ischemia by ligation the left main coronary artery with #6/0 silk string. The string was removed after 5 min of coronary occlusion to produce reperfusion. After 3 min of reperfusion, blood samples were drawn by cardiac puncture and the heart was rapidly excised. Before and during the ischemia or reperfusion period, ECGs were recorded on a PowerLab 4/25 data acquisition system (ADInstruments, Colorado Springs, Colorado) with data analysis software (Chart v4.0, ADInstruments). Ventricular arrhythmia was assessed in accordance with the definitions reported in the Lambeth Convention (12), and the incidence and duration of ventricular tachycardia (defined as 4 or more consecutive ventricular premature beats) and ventricular fibrillation (if an irregular undulating baseline was apparent) were determined. All studies were performed in accordance with the guidelines described in the national animal protection law, and the use of animal tissues was approved by the Fukuoka University Internal Review Committee.

Experimental protocols. EXPERIMENT 1. The effects of rHDL on ECG properties, BP, heart rate (HR), and cardiac function were studied. The rHDL (6 mg/kg of A-I) was injected intravenously into rats under sedation (pentobarbital, intraperitoneally). In the control group, phosphate-buffered saline (PBS) was infused instead of rHDL. Changes in ECG properties (PR, QRS, and QT intervals), BP, and HR were recorded before injection and 10 and 15 min after injection. The BP and HR were measured with a BP monitor (MK1100, Muromachi Kikai Co., Tokyo, Japan). Cardiac function was also recorded at the same time by transthoracic echocardiography (NEMIO SSA-550A, Toshiba, Tokyo, Japan). Short- and long-axis 2-dimensional views and M-mode at the level of papillary muscle were analyzed, and the ejection fraction was calculated as [100 × (volume in diastole-volume in systole)/volume in diastole].

EXPERIMENT 2. The experimental design is shown in Figure 1. Ninety-two male Wistar rats were randomly assigned to 10 groups. In the control group, PBS was infused 10 min

Abbreviations and Acronyms

ABC = adenosine triphosphate-binding cassette transporter
A-I = apolipoproteinA-I
BP = blood pressure
cGMP = cyclic guanosine monophosphate
cITP = capillary isotachophoresis
EC = endothelial cell
ECG = electrocardiogram
eNOS = endothelial nitric oxide synthesis
ERK = extracellular-signal-regulated kinase
fHDL = fast-migrating high-density lipoprotein
HCECs = human coronary artery endothelial cell
HDL = high-density lipoprotein
HR = heart rate
I/R = ischemia/reperfusion
iHDL = intermediate-migrating high-density lipoprotein
L-NAME = N-nitro-L-arginine methyl ester hydrochloride
NO = nitric oxide
NO_x = nitrite plus nitrate
PBS = phosphate-buffered saline
PI3 = phosphoinositide 3
POPC = 1-palmitoyl-2-oleoyl-phosphatidyl-choline
rHDL = reconstituted high-density lipoprotein
sHDL = slow-migrating high-density lipoprotein
SR-BI = scavenger receptor class B, type I

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before ischemic insult. The A-I, POPC, and rHDL groups were identical to the control group except that A-I, POPC, or rHDL, respectively, were infused 10 min before ischemic insult instead of PBS. The L-NAME+rHDL, PD98059+rHDL, and wortmannin+rHDL groups were identical to the rHDL group except that L-NAME, PD98059, or wortmannin, respectively, was infused 5 min before the infusion of rHDL. The L-NAME, PD98059, and wortmannin groups were identical to the L-NAME+rHDL, PD98059+rHDL, and wortmannin+rHDL groups except that rHDL was not administered. All drugs or PBS were administrated via a tail vein before coronary occlusion. After treatment, the coronary artery was occluded for 5 min and then reperfused for 3 min.

Quantification of lipoprotein subfractions by capillary isotachophoresis and agarose gel electrophoresis. Next, changes in lipoprotein subfractions after rHDL injection as characterized by capillary isotachophoresis (cITP) were examined. After tracheotomy, rats were injected with rHDL (6 mg/kg as A-I) via the tail vein. Blood samples were drawn on disodium ethylenediaminetetra-acetate before injection and after 5, 10, and 30 min from the common carotid artery. Plasma lipoprotein fractions were analyzed by cITP using a Beckman P/ACE MDQ system (Beckman-Coulter Inc., Fullerton, California), as described previously (13).

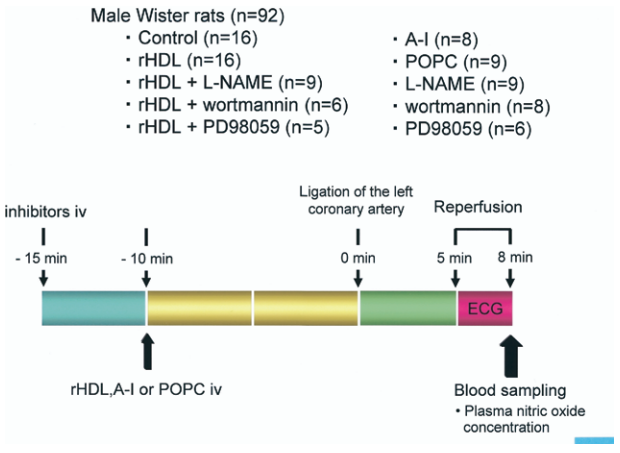

Figure 1 Experimental Protocol

Rats underwent 5 min of occlusion of the left coronary artery followed by 3 min of reperfusion. The control group was infused with phosphate-buffered saline (PBS) 10 min before occlusion. In the reconstituted high-density lipoprotein (rHDL), apolipoproteinA-I (A-I), and 1-palmitoyl-2-oleoyl-phosphatidyl-choline (POPC) groups, rHDL (6 mg/kg as A-I), A-I (6 mg/kg), or POPC (15 mg/kg) were infused instead of PBS. The L-NAME+rHDL, PD98059+rHDL, and wortmannin+rHDL groups were additionally injected with N-nitro-L-arginine methyl ester hydrochloride (L-NAME) (10 mg/kg), PD98059 (4 mg/kg), or wortmannin (16 μg/kg), respectively, 5 min before the infusion of rHDL. The L-NAME, PD98059, and wortmannin groups were identical to the L-NAME+rHDL, PD98059+rHDL, and wortmannin+rHDL groups except that rHDL was not administered. The animals were killed immediately after reperfusion for the collection of heart and blood samples. ECG = electrocardiogram; iv = intravenously.

Measurement of plasma nitrite plus nitrate levels. Plasma nitrite plus nitrate (NO_x) was determined using a NO_2/NO_3 assay kit (Cayman Chemical, Ann Arbor, Michigan).

Immunohistochemistry. After 3 min of reperfusion, some hearts were fixed in 4% paraformaldehyde and embedded in paraffin, and 3-μm sections were cut. Immunohistochemical studies were performed with commercially available polyclonal eNOS antibodies (Abcam, Cambridge, Massachusetts) and p-eNOS antibodies (Upstate Biotechnology Inc., Lake Placid, New York).

Cell culture and transfection. Human coronary artery ECs (HCECs) and Chinese hamster ovary cells (ldlA7) (14) were grown in media. In the experiments, cells supplemented without cell growth supplement were used. The complementary DNAs of adenosine triphosphate–binding cassette (ABC) transporter, ABCA1, ABCG1, and scavenger receptor class B, type I (SR-BI) cells were transfected to ldlA7 cells using the Lipofectamine 2000 liposomal reagent (Invitrogen Corporation, Carlsbad, California).

Preparation of protein extract, immunoblotting. The procedure for cell lysis and western blot analysis of signaling proteins has been described previously (15). To quantify the results for bands by densitometry, the films were scanned and the density of each band was measured using Image Gauge 4.0 (Fujifilm, Tokyo, Japan).

Statistical analysis. Results are given as the mean ± standard error. The significance of differences between mean values was evaluated by an unpaired *t* test or one-way analysis of variance followed by Fisher's protected least significant difference, as appropriate. A repeated-measures analysis of variance was also used to evaluate changes in BP, cardiac function, and ECG properties at 3 time points (before, 10 min after, and 15 min after injection) in the rHDL or control group. A value of $p < 0.05$ was considered significant.

Results

Effects of rHDL on sinus rhythm, BP, HR, and cardiac function. To analyze the acute effects of rHDL, ECG, BP, and cardiac function were recorded before injection and 10 and 15 min after injection (Table 1). Injection of rHDL caused no changes in ECG properties (PR, QRS, and QT intervals), BP, HR, or ejection fraction. Therefore, rHDL was used for further studies with I/R experiments.

Acute effects of rHDL injection on lipid profiles as determined by cITP. Plasma HDL in a healthy subject can be separated into 3 subfractions: fast-migrating HDL (fHDL), intermediate-migrating HDL (iHDL), and slow-migrating HDL (sHDL) (16). The fHDL consists only of α-migrating HDL, but the sHDL subfraction consists of both α- and pre-β-migrating HDL (17). Figure 2A shows typical cITP lipoprotein profiles in Wistar rat plasma. Rat HDL contained 4 charged HDL subfractions [fHDL (peak 1), iHDL (peaks 2 and 3), sHDL (peak 4)]. Figures 2B to

Table 1 Changes in BP, Cardiac Function, and ECG Properties in the rHDL or Control Groups

	Before	After 10 min	After 15 min	p Value
Control				
SBP (mm Hg)	119 ± 5	116 ± 9	110 ± 7	NS
HR (per min)	407 ± 27	401 ± 21	396 ± 15	NS
EF (%)	76 ± 4	75 ± 3	74 ± 2	NS
PR (ms)	50.2 ± 1.4	49.0 ± 1.7	49.7 ± 1.6	NS
QRS (ms)	13.0 ± 0.2	13.1 ± 0.2	13.2 ± 0.3	NS
QT (ms)	38.1 ± 0.9	38.6 ± 0.6	38.7 ± 1.7	NS
rHDL				
SBP (mm Hg)	110 ± 10	110 ± 9	106 ± 8	NS
HR (per min)	384 ± 7	388 ± 11	384 ± 10	NS
EF (%)	75 ± 2	76 ± 2	75 ± 1	NS
PR (ms)	50.2 ± 1.9	50.6 ± 1.9	49.8 ± 1.2	NS
QRS (ms)	13.6 ± 0.2	13.9 ± 0.2	13.9 ± 0.3	NS
QT (ms)	39.0 ± 0.4	38.8 ± 0.5	39.8 ± 1.0	NS

Values are shown as mean ± standard error.

BP = blood pressure; ECG = electrocardiogram; EF = ejection fraction; HR = heart rate; NS = not significant; rHDL = reconstituted high-density lipoprotein; SBP = systolic blood pressure.

2D show changes in HDL subfractions after rHDL injection. As shown in Figures 2B and 2C, sHDL (peak 4) had increased, while fHDL (peak 1) had decreased at 5 to 10 min after injection, and these changes persisted for almost 30 min (Fig. 2D). These results suggest that changes in lipoprotein profiles by rHDL may induce cell signaling soon after injection.

Arrhythmias during reperfusion. As shown in Figures 3A and 3B, the duration of reperfusion-induced ventricular tachycardia in the rHDL group (16.5 ± 8.8 s) was significantly decreased from its control value (45.1 ± 10.4 s). The duration of reperfusion-induced ventricular fibrillation showed a similar pattern (Fig. 3C) (rHDL group = 0 s vs. control group = 31.8 ± 10.3 s, $p < 0.01$). Using A-I or POPC alone, both of which contain the same quantity of rHDL, did not influence the duration of arrhythmias.

To assess the signaling pathways involved in the rHDL-induced suppression of arrhythmia, the rats were treated with rHDL in the presence of various inhibitors, such as PD98059, wortmannin, and L-NAME. Although the administration of PD98059, wortmannin, or L-NAME alone did not influence the duration of arrhythmia (Figs. 3B and 3C), PD98059, wortmannin, or L-NAME inhibited the antiarrhythmogenic effect of rHDL.

The eNOS pathway mediates rHDL-induced NO production. To evaluate whether rHDL increases NO production through a PI3 kinase/Akt/ERK pathway, we measured the plasma NO concentration. Plasma was drawn after 3 min of reperfusion. The rHDL significantly in-

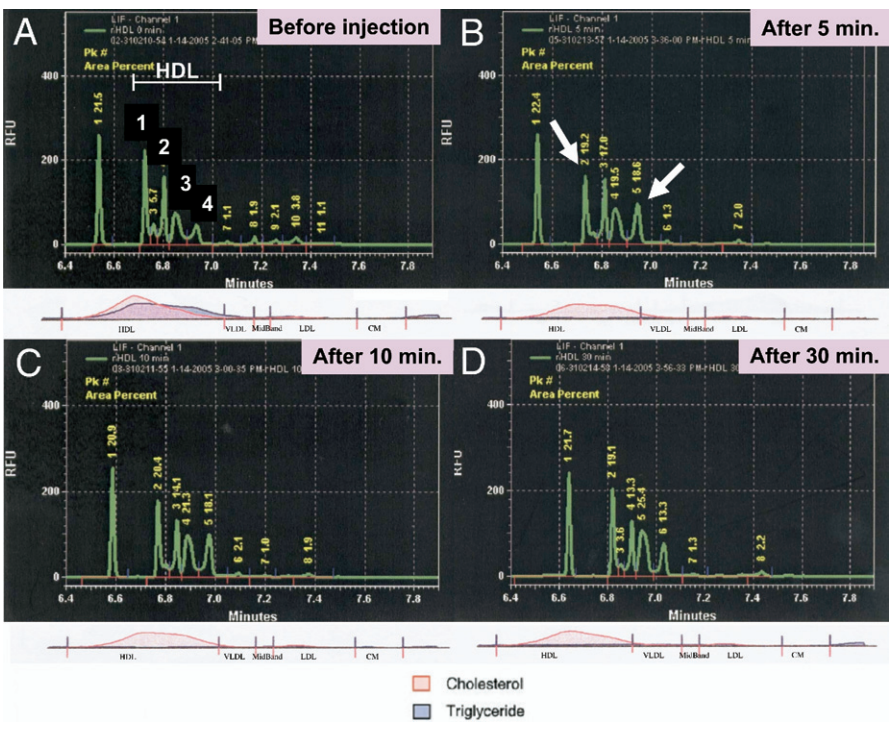

Figure 2 Lipoprotein Profiles

Lipoprotein profiles as determined by capillary isotachophoresis **(top panels)** and lipid profiles as analyzed by agarose gel electrophoresis and differential staining **(bottom panels)** in plasma from Wistar rats before rHDL injection **(A)**, and 5 min **(B)**, 10 min **(C)**, and 30 min **(D)** after rHDL injection. Peak 1, fast-migrating HDL (fHDL); peaks 2 and 3, intermediate-migrating HDL (iHDL); peaks 4, slow-migrating HDL (sHDL). Abbreviations as in Figure 1.

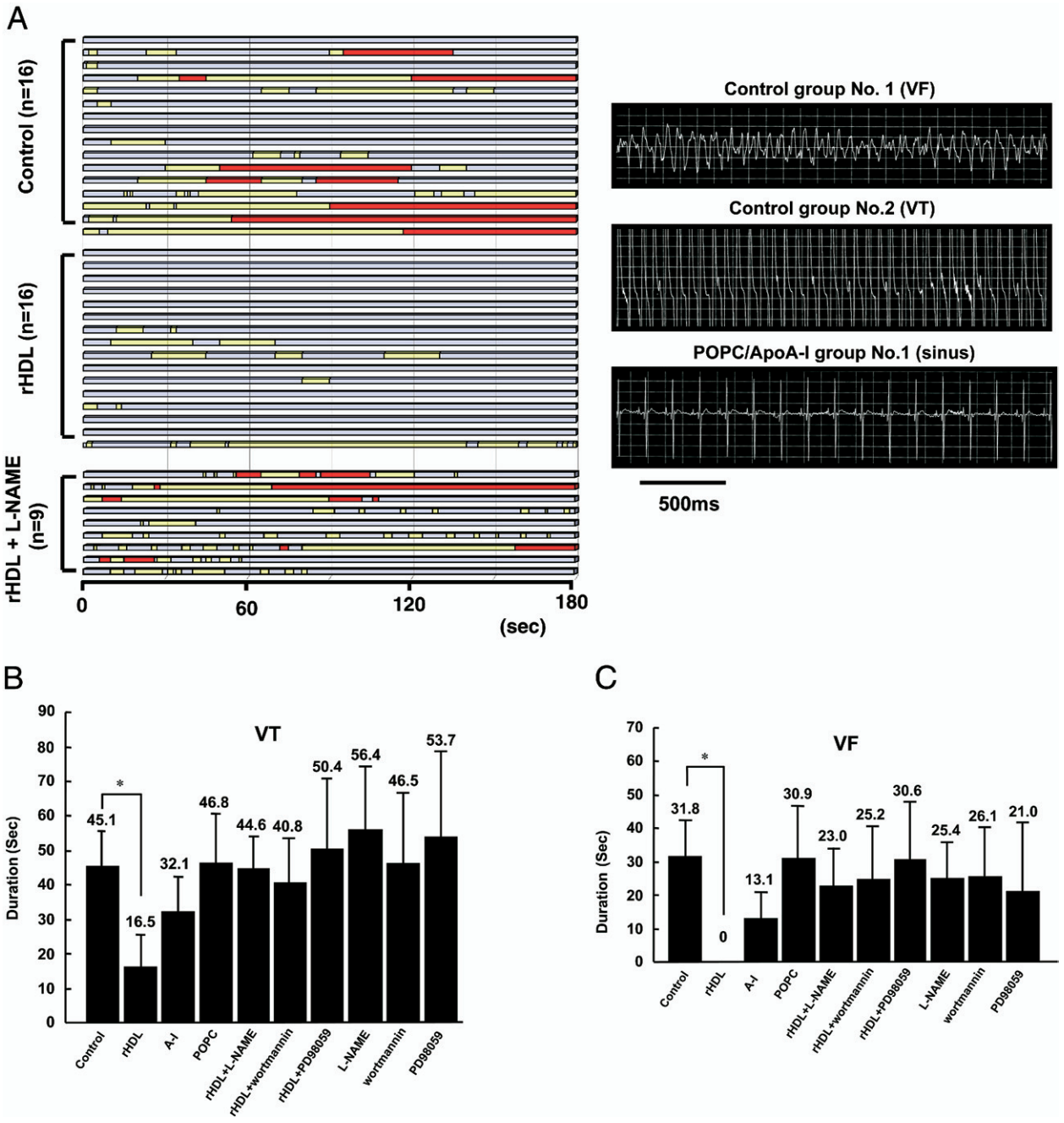

Figure 3 Effects of rHDL on Reperfusion-Induced Arrhythmia

(A) Distribution of ventricular arrhythmias during reperfusion in the control group, rHDL group, and L-NAME+rHDL group. The **orange** cells are the times during which the rat was in ventricular fibrillation (VF); the **yellow** cells show periods of ventricular tachycardia (VT); and the **gray** cells are periods during which neither VF nor VT was evident. **(B and C)** Duration (in seconds) of VT **(B)** and VF **(C)** during reperfusion. *$p < 0.05$ for the rHDL group versus the control group. Abbreviations as in Figure 1.

creased NO_x production after I/R (rHDL group 117.5 ± 6.5% relative to the control group; $p < 0.01$) (Fig. 4). The increase in NO_x production induced by rHDL was suppressed by PD98059, wortmannin, and L-NAME, suggesting that rHDL protects rats from I/R arrhythmia by producing NO through a PI3 kinase/Akt/ERK pathway.

Immunohistochemical localization of eNOS. Immunohistochemical tests for eNOS protein, using eNOS- and p-eNOS-specific antibodies, were performed on heart sections after reperfusion. The sections showed staining in coronary artery ECs and capillaries (Fig. 5A). Therefore, we used HCECs to examine the direct effect of rHDL on

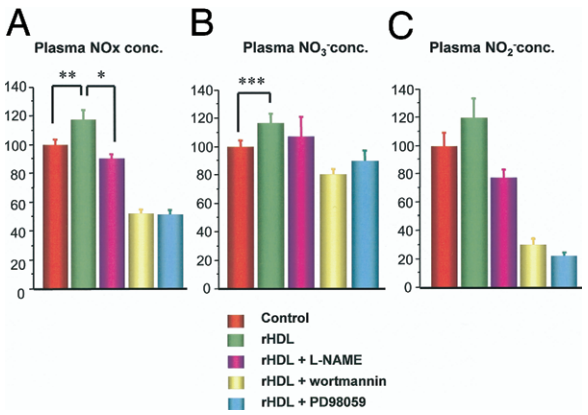

Figure 4 Plasma NO Levels

Plasma nitric oxide (NO) levels in rats: **(A)** NO_3, **(B)** NO_2, and **(C)** NO_x (NO_3+NO_2). Blood samples were drawn by cardiac puncture at the end of reperfusion, and plasma NO_3 and NO_2 levels were evaluated. The NO_3, NO_2, and NO_x levels (%) are shown relative to the control. The control sample was defined as 100% NO production, and the percent increase or decrease in NO production relative to the control was calculated for each sample. The asterisk *$p < 0.05$ for the rHDL group versus the rHDL+L-NAME group. **$p < 0.01$ for the rHDL group versus the control group. ***$p < 0.05$ for the rHDL group versus the control group. Abbreviations as in Figure 1.

eNOS activation (Fig. 5B). Treatment of HCECs with rHDL significantly stimulated p-eNOS.

Activation of Akt and ERK1/2 by rHDL in HCECs. To analyze the signal transduction from rHDL to eNOS, we measured the activation of Akt and ERK1/2 (Fig. 6A). Wortmannin, PD98059, and L-NAME alone did not affect Akt, ERK, and eNOS activation (data not shown). The rHDL induced Akt and ERK activation. The activation of Akt was blocked by wortmannin, but not by PD98059 or L-NAME (Fig. 6B). In addition, activation of ERK was blocked by wortmannin and PD98059, but not by L-NAME (Fig. 6B).

The rHDL activated p-ERK1/2 through ABCA1 and ABCG1 in ldlA7 cells. Because ldlA7 cells are an established epithelial lineage of nontransformed cells that are capable of growing under serum starvation with appropriate supplements, we used these cells as surrogate models to link rHDL to ERK in the cell membrane. To analyze what kinds of receptors mediate signaling in ldlA7 cells, we selected SR-BI, ABCA1, and ABCG1 (Fig. 6C) because HCECs, but not ldlA7 cells, endogenously express these receptors by reverse transcription-polymerase chain reaction (data not shown). Although rHDL induced ERK activation in HCECs, rHDL did not activate ERK in ldlA7 cells because there may be differences in the endogenous expression of receptors or transporters between HCECs and ldlA7 cells. The rHDL in ABCA1- and ABCG1-transfected ldlA7 cells significantly stimulated p-ERK compared to mock-transfected or SR-BI-transfected cells.

Discussion

The main finding of the present study is that rHDL-induced NO production, probably through an Akt/ERK pathway in ECs, may suppress reperfusion-induced arrhythmias in vivo. Furthermore, although HDL activates eNOS via SR-BI or sphingosine 1-phosphate (18), we found that rHDL may activate eNOS through ABCA1 or ABCG1 in ECs.

An important consequence of myocardial ischemia and reperfusion is the occurrence of cardiac dysrhythmias. In our in vivo study, rHDL increased NO production and suppressed I/R arrhythmia. The abundance of NO in the rHDL-treated rat does not simply reflect the plasma NO concentration alone, and several mechanisms are related to the increase in plasma NO. First, NO can also be generated in the ischemic heart by the direct reduction of nitrite to NO under acidotic and highly reduced conditions (19). Second, NO is scavenged by hemoglobin after generation (20). These mechanisms may be related to the increase in plasma NO after rHDL injection in this study. Although the changes in plasma NO by rHDL are quite small, many

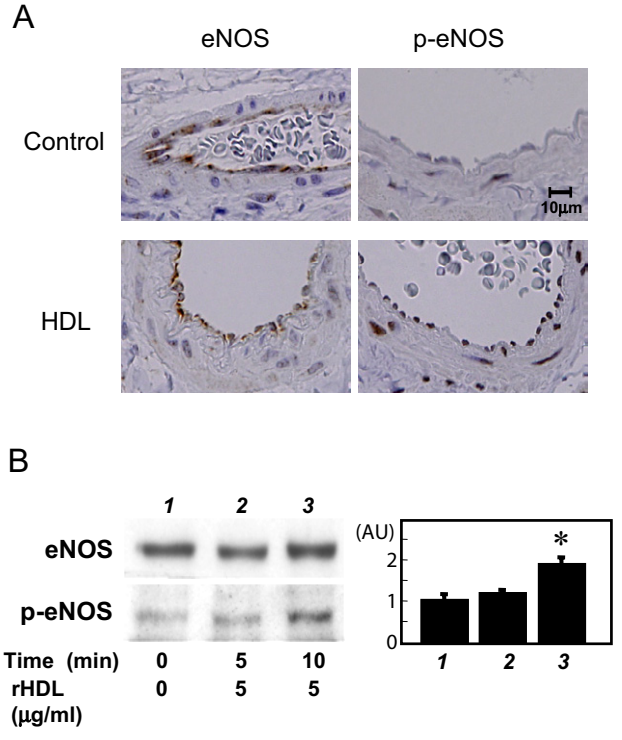

Figure 5 Localization and Expression Levels of eNOS

(A) Immunohistochemical staining for endothelial nitric oxide synthase (eNOS) and phospho-eNOS (p-eNOS) from rat hearts after reperfusion in the control and rHDL groups. Representative sections are shown (magnification ×800). **(B)** The rHDL activated p-eNOS after 10 min in human coronary artery endothelial cells. Representative pictures are shown. The graph shows the ratio of p-eNOS to total eNOS relative to 1.0 for no treatment. *Significant increase ($p < 0.05$) versus no treatment. Abbreviations as in Figure 1.

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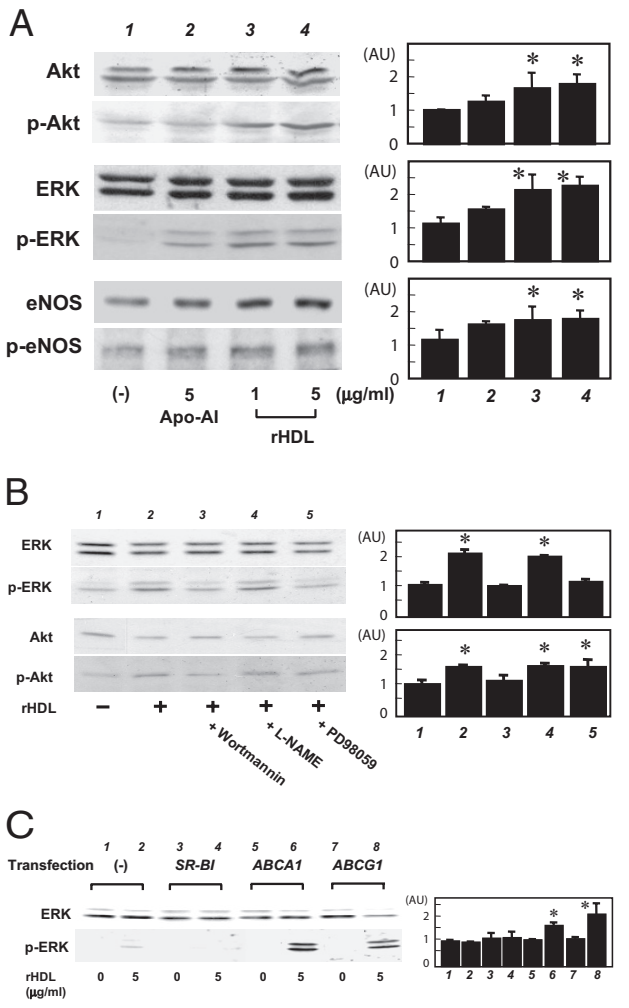

Figure 6 **Expression Levels of Akt, ERK, and eNOS**

(A) The rHDL activated p-Akt, p-ERK, and p-eNOS after 10 min of incubation in human coronary artery endothelial cells. **(B)** The rHDL-induced extracellular-signal-regulated kinase (ERK) activation was blocked by wartmannin and PD98059, but not L-NAME. The rHDL (5 μg/ml)-induced Akt activation was blocked by wartmannin (100 nmol/l), but not by PD98059 (3 μmol/l) or L-NAME (1 μmol/l). **(C)** The scavenger receptor class B, type I (SR-BI), adenosine triphosphate-binding cassette transporter (ABC) A1, or ABCG1 cells were transfected into IdlA7 cells. Forty-eight hours after transfection, the cells were cultured under serum-free conditions for an additional 24 h. The rHDL (5 μg/ml) was added to the medium for 10 min, and then the cells were lysed and analyzed. Representative pictures are shown **(A to C).** The **graph** shows the ratio of phospho- to total eNOS, Akt, and ERK in each sample relative to 1.0 for no treatment. *Significant increase ($p < 0.05$) versus no treatment. Abbreviations as in Figure 1.

studies have suggested that low doses of NO may be beneficial, but high doses are harmful in ischemia reperfusion (21). Physiologically low concentrations of NO reduce leukocyte adhesion to endothelium, suppress inflammation, and increase contractility, but high concentrations cause depression of cardiac function by reacting with superoxide and forming the toxic product peroxynitrite (22). In addition, in our study, the change in plasma NO caused by rHDL was small, and this effect of rHDL was abrogated by L-NAME. Therefore, NO may be a primary mediator. These results suggest that the production of NO by rHDL results from activation of the Akt/ERK/NO pathway, and these effects are related to the suppression of arrhythmia.

We also found that injection of rHDL leads to an increase in sHDL and a decrease in fHDL within 5 min in vivo. This finding agrees with our previous in vitro finding that both fHDL and iHDL were converted to sHDL after the incubation of plasma with rHDL (23). The fHDL consisted of only α-migrating HDL, but the sHDL subfraction consisted of both α- and pre-β-migrating HDL (17). In addition, these findings also agree with the hypothesis of Nanjee et al. (24) that the generation of small pre-β-HDL in vivo results from the fusion of rHDL with endogenous α-HDL (24). Because lipid-free A-I and discoidal HDL both have pre-β electrophoretic mobility when subjected to agarose gel electrophoresis (25), newly generated pre-β-HDL after the injection of rHDL may contain both of these forms of HDL. Because the process that promotes HDL particle fusion has the capacity to promote the dissociation of lipid-poor A-I (25), the fusion of rHDL with α-HDL may lead to the dissociation of lipid-poor A-I. Our in vivo experiments show that these processes occurred very quickly (within <10 min), continued for at least 30 min, and may be effective for inducing cell signaling.

Both the Akt and ERK signaling are necessary for the HDL-mediated stimulation of eNOS enzymatic activity (26). Although the phosphorylation of PI3 kinase by rHDL may induce the parallel activation of Akt and ERK, which leads to eNOS activation, rHDL-induced ERK activation may be mediated by ABCA1 or ABCG1, but not by SR-BI. We speculated that rHDL activates eNOS through ABCA1 and ABCG1. Both ABCA1 and ABCG1 are transporters that regulate cholesterol and phospholipid export to HDL. However, there have been several reports that ABC transporters are implicated in signal transduction (27). Because a lipid-free form of A-I (pre-β-HDL) interacts with ABCA1 and mature HDL interacts with ABCG1 (28), after injection of rHDL, the newly developed pre-β-HDL and mature HDL may interact with ABCA1 and ABCG1, respectively. However, rHDL may also interact directly with ABCA1 and ABCG1. Although newly developed pre-β-migrating discoidal HDL and mature HDL may interact with SR-BI (1) or sphingosine 1-phosphate 3 (29), these may not contribute to ERK activation in this study.

The pathogenesis of I/R injury and arrhythmia involves many mechanisms (30). Recently, a growing body of evidence has suggested that high levels of cytosolic calcium play an important role in I/R arrhythmia (31). Both endogenously generated NO, as well as NO donors, have a protective effect against I/R arrhythmia (32). The NO is also known to trigger ischemic preconditioning against I/R arrhythmia (8). Several possible mechanisms may underlie the beneficial effects of NO on arrhythmias.

The NO increases cyclic guanosine monophosphate (cGMP) levels through the activation of guanylyl cyclase (33). The NO-induced production of cGMP in the myocardium plays an important role in the antiarrhythmic effect of NO (34). The increase in myocardial cGMP inhibits Ca^{2+} influx through L-type Ca channels and reduces Ca^{2+} overload during I/R (35). In addition, increased cGMP and inhibition of Ca^{2+} influx by NO may lead to coronary vasodilation (36). Thus, NO may prevent arrhythmias by reducing intracellular Ca^{2+} overload and subsequent coronary vasodilation.

Because NO also has an antioxidative effect, this effect may play a role in the antiarrhythmogenic activity of rHDL. In this study, the administration of rHDL and the resulting increase in NO may reduce I/R injury (37) but not myocardial infarct size, because no infarct would result from the short occlusion. In fact, in our in vivo model of rat myocardial infarction, there was a significant reduction in infarct size with rHDL administration once per week for 4 weeks (38).

Although several medications are used clinically, such as statins and fibrates that increase HDL, there is no clear evidence that these drugs are efficacious toward reperfusion-induced arrhythmias. However, many clinical trials have shown that these drugs reduced total cardiac events as well as coronary events, and such a reduction of total cardiac events may include the reduction of sudden death caused by reperfusion-induced arrhythmia.

Study limitations. There are 3 important limitations in this study. One possible limitation of this work is that the animal model of diseases and its treatment may not precisely represent the human pathology and treatment. Although rHDL was administered before ischemia and was given only once and for a short period, the administration of rHDL after ischemia or a more sufficient administration may be important for elucidating the optimal clinical application and precise mechanisms of rHDL. Second, although we did not measure the core temperature of the rats, we can presume from other studies that the core temperature of pentobarbital-anesthetized rats in this study must be lowered (39). However, we compared the arrhythmias in all groups under the same conditions; that is, we kept the rats at a stable room temperature (23°C) and placed the rats on an expanded polystyrene table to avoid a decrease in temperature during the experiments. Third, to evaluate the development of NO in cardiac tissue and coronary arteries, we needed to directly investigate the level of eNOS in the heart. It was very difficult to compare the changes in the level of eNOS activity in the heart. Sections from rat heart showed eNOS and p-eNOS staining predominantly in coronary artery ECs and capillaries. In addition, we examined the effect of rHDL on primary cultures of rat myocytes. However, rHDL had no effect on eNOS activation in myocytes (data not shown). Therefore, we thought that rHDL activates eNOS in ECs but not in myocytes in the heart.

Conclusions

In summary, we have demonstrated that rHDL suppresses I/R arrhythmia presumably by increasing NO production. The rHDL-induced NO production, probably mediated by ABCA1 or ABCG1 through an Akt/ERK/NO pathway in ECs, may suppress reperfusion-induced arrhythmias. This study suggests the potential of infusing rHDL particles as a therapeutic strategy against cardiac sudden death after I/R.

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Reprint requests and correspondence: Dr. Shin-ichiro Miura, Department of Cardiology, Fukuoka University School of Medicine, 7-45-1 Nanakuma, Jonan-ku, Fukuoka, 814-0180, Japan. E-mail: miuras@cis.fukuoka-u.ac.jp.

REFERENCES

1. Linsel-Nitschke P, Tall AR. HDL as a target in the treatment of atherosclerotic cardiovascular disease. Nat Rev Drug Discov 2005;4:193–205.
2. Wannamethee G, Shaper AG, Macfarlane PW, Walker M. Risk factors for sudden cardiac death in middle-aged British men. Circulation 1995;91:1749–56.
3. Burke AP, Farb A, Malcom GT, Liang YH, Smialek J, Virmani R. Coronary risk factors and plaque morphology in men with coronary disease who died suddenly. N Engl J Med 1997;336:1276–82.
4. Liu YB, Wu CC, Lee CM, et al. Dyslipidemia is associated with ventricular tachyarrhythmia in patients with acute ST-segment elevation myocardial infarction. J Formos Med Assoc 2006;105:17–24.
5. Mochlzuki S, Okumura M, Tanaka F, et al. Ischemia-reperfusion arrhythmias and lipids: effect of human high- and low-density lipoproteins on reperfusion arrhythmias. Cardiovasc Drugs Ther 1991;5:269–76.
6. Kuvin JT, Alsheikh-Ali AA, Karas RH. High-density lipoprotein cholesterol-raising strategies. J Cardiovasc Pharmacol 2006;47:196–204.
7. Calabresi L, Rossoni G, Gomaraschi M, Sisto F, Berti F, Franceschini G. High-density lipoproteins protect isolated rat hearts from ischemia-reperfusion injury by reducing cardiac tumor necrosis factor-alpha content and enhancing prostaglandin release. Circ Res 2003;92:330–7.
8. Vegh A, Komori S, Szekeres L, Parratt JR. Antiarrhythmic effects of preconditioning in anaesthetised dogs and rats. Cardiovasc Res 1992;26:487–95.
9. Yahiro E, Ideishi M, Wang LX, et al. Reperfusion-induced arrhythmias are suppressed by inhibition of the angiotensin II type 1 receptor. Cardiology 2003;99:61–7.
10. Jalowy A, Schulz R, Heusch G. AT1 receptor blockade in experimental myocardial ischemia/reperfusion. J Am Soc Nephrol 1999;10 Suppl 11:S129–36.
11. Rye KA, Garrety KH, Barter PJ. Changes in the size of reconstituted high density lipoproteins during incubation with cholesteryl ester transfer protein: the role of apolipoproteins. J Lipid Res 1992;33:215–24.
12. Walker MJ, Curtis MJ, Hearse DJ, et al. The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia infarction, and reperfusion. Cardiovasc Res 1988;22:447–55.
13. Zhang B, Matsunaga A, Saku K, Nakano S, Yamada T. Associations among plasma lipoprotein subfractions as characterized by analytical

capillary isotachophoresis, apolipoprotein E phenotype, Alzheimer disease, and mild cognitive impairment. Arterioscler Thromb Vasc Biol 2004;24:e144–6.
14. Kingsley DM, Krieger M. Receptor-mediated endocytosis of low density lipoprotein: somatic cell mutants define multiple genes required for expression of surface-receptor activity. Proc Natl Acad Sci U S A 1984;81:5454–8.
15. Miura S, Matsuo Y, Saku K. Transactivation of KDR/Flk-1 by the B2 receptor induces tube formation in human coronary endothelial cells. Hypertension 2003;41:1118–23.
16. Bottcher A, Schlosser J, Kronenberg F, et al. Preparative free-solution isotachophoresis for separation of human plasma lipoproteins: apolipoprotein and lipid composition of HDL subfractions. J Lipid Res 2000;41:905–15.
17. Schmitz G, Mollers C, Richter V. Analytical capillary isotachophoresis of human serum lipoproteins. Electrophoresis 1997;18:1807–13.
18. Nofer JR, van der Giet M, Tolle M, et al. HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. J Clin Invest 2004;113:569–81.
19. Zweier JL, Wang P, Samouilov A, Kuppusamy P. Enzyme-independent formation of nitric oxide in biological tissues. Nat Med 1995;1:804–9.
20. Kim-Shapiro DB, Schechter AN, Gladwin MT. Unraveling the reactions of nitric oxide, nitrite, and hemoglobin in physiology and therapeutics. Arterioscler Thromb Vasc Biol 2006;26:697–705.
21. Jugdutt BI. Nitric oxide and cardioprotection during ischemia-reperfusion. Heart Fail Rev 2002;7:391–405.
22. Stangl V, Baumann G, Stangl K, Felix SB. Negative inotropic mediators released from the heart after myocardial ischaemia-reperfusion. Cardiovasc Res 2002;53:12–30.
23. Zhang B, Miura S, Fan P, et al. ApoA-I/phosphatidylcholine discs remodels fast-migrating HDL into slow-migrating HDL as characterized by capillary isotachophoresis. Atherosclerosis 2006;188:95–101.
24. Nanjee MN, Doran JE, Lerch PG, Miller NE. Acute effects of intravenous infusion of ApoA1/phosphatidylcholine discs on plasma lipoproteins in humans. Arterioscler Thromb Vasc Biol 1999;19:979–89.
25. Rye KA, Barter PJ. Formation and metabolism of prebeta-migrating, lipid-poor apolipoprotein A-I. Arterioscler Thromb Vasc Biol 2004; 24:421–8.
26. Mineo C, Yuhanna IS, Quon MJ, Shaul PW. High density lipoprotein-induced endothelial nitric-oxide synthase activation is mediated by Akt and MAP kinases. J Biol Chem 2003;278: 9142–9.
27. Haidar B, Denis M, Marcil M, Krimbou L, Genest J Jr. Apolipoprotein A-I activates cellular cAMP signaling through the ABCA1 transporter. J Biol Chem 2004;279:9963–9.
28. Rader DJ. Molecular regulation of HDL metabolism and function: implications for novel therapies. J Clin Invest 2006;116:3090–100.
29. Matsuo Y, Miura SI, Kawamura A, Uehara Y, Rye KA, Saku K. Newly developed reconstituted high-density lipoprotein containing sphingosine-1-phosphate induces endothelial tube formation. Atherosclerosis 2007:194:159–68.
30. Moens AL, Claeys MJ, Timmermans JP, Vrints CJ. Myocardial ischemia/reperfusion-injury, a clinical view on a complex pathophysiological process. Int J Cardiol 2005;100:179–90.
31. Ravens U, Himmel HM. Drugs preventing Na+ and Ca2+ overload. Pharmacol Res 1999;39:167–74.
32. Kubota I, Han X, Opel DJ, et al. Increased susceptibility to development of triggered activity in myocytes from mice with targeted disruption of endothelial nitric oxide synthase. J Mol Cell Cardiol 2000;32:1239–48.
33. Russwurm M, Koesling D. NO activation of guanylyl cyclase. Embo J 2004;23:4443–50.
34. Pabla R, Bland-Ward P, Moore PK, Curtis MJ. An endogenous protectant effect of cardiac cyclic GMP against reperfusion-induced ventricular fibrillation in the rat heart. Br J Pharmacol 1995;116: 2923–30.
35. Mery PF, Lohmann SM, Walter U, Fischmeister R. Ca2+ current is regulated by cyclic GMP-dependent protein kinase in mammalian cardiac myocytes. Proc Natl Acad Sci U S A 1991;88:1197–201.
36. Luscher TF. Endothelium-derived nitric oxide: the endogenous nitrovasodilator in the human cardiovascular system. Eur Heart J 1991;12 Suppl E:2–11.
37. Rossoni G, Manfredi B, De Gennaro Colonna V, et al. Nitric oxide and prostacyclin pathways: an integrated mechanism that limits myocardial infarction progression in anaesthetized rats. Pharmacol Res 2006;53:359–66.
38. Kiya Y, Miura S, Imaizumi S, et al. Reconstituted high-density lipoprotein prevents pathological remodeling after myocardial infarction (abstr). Circ J 2005;69:646.
39. Kiyatkin EA, Brown PL. Brain and body temperature homeostasis during sodium pentobarbital anesthesia with and without body warming in rats. Physiol Behav 2005;84:563–70.